Targeting Tumor-Resident Myeloid Cells Via BTK Inhibition to Enhance Oncolytic Viroimmunotherapy

Undergraduate Research Thesis

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by

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Abstract

Pediatric sarcomas are highly aggressive cancers that metastasize quickly and can return after treatment. Despite the use of harsh treatments, survival rates for older children and those with metastatic disease remain low. Oncolytic virotherapy is a promising treatment option for pediatric sarcomas that aims to destroy cancer cells and induce antitumor immunity using live, attenuated viruses such as herpes simplex-1 viruses (oHSV). Virotherapy, however, also stimulates the recruitment of tumor-associated immunosuppressive cells, like myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM), to the tumor microenvironment. Such immunosuppressive cells limit the ability of the virus to replicate and spread. We hypothesize that this recruitment results in reduced therapeutic efficacy.

Previous studies show that trabectedin, a chemotherapeutic, in combination with oHSV treatment enhances the efficacy of virotherapy in A673 Ewing sarcoma models. This is likely due to trabectedin's ability to suppress TAM and MDSC recruitment to the tumor microenvironment. We termed this combination "myelolytic-virotherapy." Though trabectedin was able to enhance oHSV virotherapy through the reduction of immunosuppressive cell recruitment, this mechanism is not well understood. Thus, there is a need to understand if solely targeting tumor-resident MDSC enhances virotherapy. By determining if targeting MDSC improves the efficacy of oncolytic virotherapy, we can develop novel myelolytic-virotherapies that have increased therapeutic efficacy in multiple tumor models. This could have great potential for pediatric cancer patients.

Previous studies have also shown that the efficacy of oHSV virotherapy may be dependent on virus-induced antitumor T cell responses. Treatment with oHSV enhanced overall survival and induced T cell recruitment to the tumor microenvironment in M3-9-M

rhabdomyosarcoma models. Therefore, there is an additional need to investigate whether antitumor T cell responses are necessary to enhance the efficacy of oncolytic virotherapy, or if solely suppressing MDSC is enough.

A study conducted by Dr. William Carson found that ibrutinib, an irreversible Bruton's tyrosine kinase (BTK) inhibitor, selectively reduces MDSC recruitment to tumors and limits their ability to suppress T cell proliferation. Ibrutinib also improved the efficacy of immune based cancer therapies. We hypothesize that ibrutinib enhances oncolytic virotherapy in Ewing sarcoma and rhabdomyosarcoma models by inhibiting tumor-associated MDSC recruitment and enhancing viral replication. In this study, we performed cellular infiltrate and viral replication studies in A673 Ewing sarcoma models to evaluate the effect of ibrutinib on MDSC recruitment and oHSV replication *in vivo*. We also performed survival studies in A673 and M3-9-M tumor-bearing mice to determine the effect of ibrutinib on tumor growth and survival. The results indicate that while ibrutinib does reduce MDSC recruitment to the microenvironment, it does not enhance oHSV replication or improve the efficacy of oHSV virotherapy.

Introduction

Pediatric sarcomas are aggressive types of childhood cancer that are often high-grade, fast spreading, and can recur after treatment [1]. Ewing sarcoma and rhabdomyosarcoma are two of the most common types of pediatric sarcomas [2]. While cure rates are relatively high for young patients with localized disease, the survival rate remains low for children who are older or experience tumor metastasis [3].

Most pediatric sarcomas are treated using a multimodal approach that includes surgery, chemotherapy, and radiation [4]. These therapies, however, are harsh and often have late terms side effects that increase morbidity and mortality rates for childhood cancer survivors. Some of these side effects include growth hormone deficiency and risk of developing secondary cancers [5]. Therefore, there is a need for more targeted therapies that both limit long-term side effects and increase survival outcomes for pediatric sarcoma patients.

Oncolytic virotherapy is a promising potential therapy for pediatric sarcomas. Oncolytic virotherapy uses live, attenuated viruses, like herpes-simplex viruses, to infect and destroy tumor cells via lysis and activate the immune system's antitumor responses [6]. Multiple clinical studies have shown that this form of therapy may require lower doses, result in less toxicity, and cause fewer long-term side effects in pediatric patients [7]. HSV1716, also known as Seprehvir, has been shown in phase I clinical trials to be safe in pediatric populations, making it a good choice for this study [8].

In addition to killing cancer cells and activating the body's immune response, however, oncolytic viruses can also recruit immunosuppressive cells to the tumor microenvironment. Such immunosuppressive cells, like MDSC, hinder the efficacy of virotherapy by limiting the virus' ability to replicate and spread. Given the potential of oncolytic virotherapy to lower the risk of

side effects in childhood cancer patients, there is a need to investigate if blocking the recruitment of these immunosuppressive cells increases therapeutic efficacy. Ibrutinib, an irreversible BTK inhibitor, reduces the presence of MDSC in the tumor area and may therefore improve the efficacy of oHSV therapy. The development of targeted treatments that combine oHSV virotherapy with drugs like ibrutinib could be very beneficial for pediatric sarcoma patients.

Focus of the Research

The purpose of this research is to determine if using ibrutinib to target myeloid derived suppressor cells via BTK inhibition enhances oncolytic virotherapy in pediatric sarcoma tumor models.

Significance of the Research

Oncolytic virotherapy is an exciting new therapy that can be used to kill tumor cells and initiate an antitumor immune response in patients of all ages. In 2015, the FDA approved the first oncolytic virotherapy, Imlygic, after the successful completion of phase III clinical trials in adults with melanoma [9]. Phase I clinical trials have shown that oHSV therapy is also safe and well tolerated by children, which suggests it may be a good therapy choice for young populations [10]. Because it is safe, directly targets cancerous cells, and creates an immune response, oncolytic virotherapy could become an effective therapy for many different cancers and patient populations. Developing a better understanding of virotherapy and its impact on the tumor microenvironment is key to making it more effective for individuals with cancer.

Another benefit of virotherapy is its potential to limit the long-term side effects that other common cancer treatments cause in pediatric patients. As the number of childhood cancer survivors continues to grow, the long-term effects of aggressive cancer therapies have become more apparent. Previous studies have shown that up to 50% of childhood cancer survivors will experience late effects from treatment, often decades after they are cured [11]. This makes the creation of targeted therapies with lower doses and fewer side-effects for children even more important. Pediatric sarcoma patients, who often receive high doses of both chemotherapy and radiation, may greatly benefit from oncolytic virotherapy.

Research projects like this one are important because pediatric cancers are understudied, as is the tumor microenvironment's influence on virotherapy. Few people, if any, are studying the studying the combination of myelolytic-virotherapy in pediatric tumor models. By acquiring a better understanding of the relationship between oncolytic viruses, the tumor microenvironment, and tumor-associated myeloid cells, we will be able to create novel

treatments for enhancing viroimmunotherapy that can be readily translated to the clinic. These myelolytic-virotherapies could have positive and far-reaching implications for patients of all ages and a variety of tumor types.

Definition of Key Terms

A673: A human Ewing sarcoma cell line.

Athymic nude mice: Mice that lack a fully developed thymus. As a result, they are T cell deficient and immunodeficient.

B6-albino mice: C57BL/6J mice that have a mutation in the tyrosinase gene. As a result, they lack pigment in their eyes, skin, and hair.

Bruton's tyrosine kinase: A tyrosine kinase that plays a key role in myeloid cell development, function, and signaling.

$Cd11b^+$: A myeloid cell marker.

$Cd11c^+$: A pan dendritic cell marker.

Ewing sarcoma: An aggressive childhood cancer that most frequently occurs in the bone and soft tissue. It is the second most common form of pediatric bone cancer.

gMDSC: Granulocytic myeloid-derived suppressor cell.

HSV1716: An oncolytic herpes simplex virus-1 being used in adult and pediatric cancer clinical trials. It has the *RL1* gene deleted, which encodes ICP34.5, a neurovirulence factor.

Ibrutinib: An irreversible Bruton's tyrosine kinase inhibitor that selectively reduces MDSC recruitment to the tumor microenvironment. It is an FDA-approved immunotherapy drug that is clinically used for B cell malignancies.

M2-like TAM: Pro-tumor macrophages that suppress the host antitumor and antiviral immune responses.

M3-9-M: A murine rhabdomyosarcoma cell line.

mMDSC: Monocytic myeloid-derived suppressor cell.

Myeloid-derived suppressor cell (MDSC): Immature myeloid cells that suppress the immune system and aid in tumor evasion.

NK: Natural killer cell.

oHSV: Oncolytic herpes simplex virus.

Oncolytic virotherapy: A type of cancer therapy that uses live, attenuated viruses to directly infect and lyse tumor cells and activate a host antitumor immune response.

Programmed cell death protein (PD)-1: A T cell exhaustion marker that plays a role in T cell suppression. It is an effective therapy target for tumors that express its ligands, PD-L1 and PD-L2.

Rhabdomyosarcoma: A type of childhood cancer that begins in the soft tissue. It is the most common form of pediatric soft tissue sarcoma.

Tumor-associated macrophage (TAM): Macrophages that have been recruited to the tumor microenvironment and play a role in tumor development and suppression of the immune system.

Trabectedin: Chemotherapy drug that reduces the presence of TAM and MDSC in the tumor microenvironment.

Background

The efficacy of oncolytic virotherapy is dependent on both the ability of the virus to stimulate the host immune system's antitumor mechanisms and the tumor microenvironment's influence on immune response [12]. Previous studies have shown that virotherapy alone is only moderately effective in slowing tumor growth in Ewing sarcoma models, partly due to $Cd11b^+$ myeloid cell infiltration [13]. MDSC are known immunosuppressors that promote tumor progression. By suppressing the immune response created by oncolytic virotherapy, MDSC hinder its therapeutic benefits [14]. To study the effects of MDSC on oncolytic virus efficacy in Ewing sarcoma models, A673 xenograft-bearing athymic nude mice were treated with two doses of trabectedin in combination with two doses of oHSV. Trabectedin is an FDA-approved chemotherapeutic agent that selectively depletes TAM and MDSC. The results of this study showed that while oHSV increased the percentage of TAM and MDSC in the cellular infiltrate, trabectedin reduced both cell populations. The combination of trabectedin and oHSV resulted in the greatest reduction of myeloid cells. In A673 Ewing sarcoma mouse models, the combination therapy significantly improved the survival percentage of mice compared to oHSV treatment alone, resulting in a 78% response rate overall [15]. While trabectedin significantly improved the efficacy of oHSV virotherapy, it is unclear if this effect is due to the suppression of both TAM and MDSC, or MDSC alone. Given these findings, it is necessary to investigate whether solely targeting MDSC is enough to enhance oncolytic virotherapy.

A recent study showed that ibrutinib, an FDA-approved irreversible Bruton's tyrosine kinase inhibitor, selectively reduces MDSC recruitment to the tumor microenvironment. Immunocompetent mice bearing EMT6 murine mammary carcinoma tumors received daily doses of ibrutinib or vehicle. At the conclusion of the study, the MDSC population in the tumors was analyzed, and ibrutinib treatment significantly reduced the $CD11b^+/GR\text{-}1^+$ population.

Ibrutinib treatment did not significantly reduce tumor growth, but it did improve the efficacy of immune based cancer therapies. A combination treatment of ibrutinib and anti-PD-L1 antibody resulted in a significant reduction in tumor growth in EMT6 mammary carcinoma tumor-bearing Balb/c mice [16]. Ibrutinib's ability to reduce tumor-associated MDSC recruitment and enhance the efficacy of cancer immunotherapies suggests that the combination of ibrutinib and oHSV treatment would reduce tumor growth and increase the efficacy of oHSV virotherapy in Ewing sarcoma models.

In addition to suppressing the recruitment of tumor-associated MDSC, ibrutinib also reduced the ability of MDSC to suppress T cell proliferation [16]. Oncolytic virotherapy induces antitumor T cell immunity, and its efficacy may rely on an antitumor T cell response [17]. Given this information, ibrutinib may enhance oncolytic virotherapy by both suppressing MDSC and enhancing T cell proliferation.

There are currently no immunocompetent Ewing sarcoma animal models, so *in vivo* studies must be conducted in immunocompromised mice. Because these mice lack a developed thymus, they cannot be used to study the relationship between ibrutinib, oHSV virotherapy, and antitumor T cell responses. Previous studies have shown that HSV1716 virotherapy slows tumor growth and enhances survival in immunocompetent, M3-9-M tumor-bearing mice [17]. The effects of HSV1716 virotherapy were dependent on a virus-induced antitumor T cell response. Given these findings, M3-9-M is an appropriate model for investigating whether the effect of ibrutinib on oncolytic virotherapy efficacy is dependent on enhanced T cell proliferation.

Research Aims and Hypotheses

Aim 1: Determine the effect of ibrutinib on the composition of Ewing sarcoma tumor cellular infiltrates. An analysis of the tumor cellular infiltrate from A673 tumors treated with PBS control, HSV1716, ibrutinib, or a combination of ibrutinib and HSV1716 will be performed using flow cytometry. We expect that that A673 tumors treated with ibrutinib or the combination treatment will exhibit a reduced number of MDSC in comparison to the PBS control and HSV1716 groups. We hypothesize that ibrutinib will reduce the presence of MDSC in the Ewing sarcoma tumor cellular infiltrate by inhibiting Bruton's tyrosine kinase.

Aim 2: Determine the effectiveness of the combination of ibrutinib and HSV1716 virotherapy in causing tumor regressions and increasing survival in Ewing sarcoma. A survival study in A673 xenograft-bearing nude mice will be performed using PBS control, HSV1716, ibrutinib, or a combination of ibrutinib and HSV1716. We expect that the combination therapy will result in the greatest number of tumor regressions, increase survival, and have the greatest therapeutic efficacy compared to ibrutinib or HSV1716 treatment alone. We hypothesize that the combination of ibrutinib and HSV1716 treatment will cause tumor regressions and increase survival in Ewing sarcoma models by reducing MDSC recruitment to the tumor microenvironment and activating innate antitumor mechanisms.

Aim 3: Determine the effect of ibrutinib treatment on viral replication in HSV1716-treated Ewing sarcoma tumors. Serial plaque assays will be conducted using the lysates from A673 tumors treated with HSV1716 or a combination of ibrutinib and HSV716. We expect that the combination therapy will result in increased viral replication compared to HSV1716 alone. We hypothesize that ibrutinib will enhance viral replication in HSV1716-treated Ewing sarcoma

tumors by reducing MDSC recruitment to the tumor microenvironment, thus allowing for increased viral production and spread.

Aim 4: Determine the effectiveness of the combination of ibrutinib and HSV1716 virotherapy in causing tumor regressions and increasing survival in rhabdomyosarcoma. A survival study in M3-9-M tumor-bearing B6-albino mice will be performed using PBS control, HSV1716, ibrutinib, or a combination of ibrutinib and HSV1716. We expect that the combination therapy will result in the greatest number of tumor regressions, increase survival, and have the greatest therapeutic efficacy compared to ibrutinib or HSV1716 treatment alone. We hypothesize that the combination of ibrutinib and HSV1716 treatment will cause tumor regressions and increase survival in rhabdomyosarcoma models by reducing MDSC recruitment to the tumor microenvironment and activating innate antitumor mechanisms, including antitumor T cell proliferation.

Methodology

Aim 1: Cellular infiltrate analysis. To determine the effect of ibrutinib on the composition of the tumor cellular infiltrate in A673 tumors, cellular infiltrate analysis using flow cytometry was performed. 5.0 x 10^6 A673 cells in a 2:1 150-uL mix of PBS and matrigel were injected subcutaneously into the flanks of athymic nude mice. After tumors reached a volume between 88.6-1008.8 mm^3, the mice (n=2-3) were randomized into one of four study groups: PBS control, HSV1716, ibrutinib, or a combination of HSV1716 + ibrutinib. Most tumor volumes were between 300-600 mm^3. Two 1x10^7 pfu doses of HSV1716 were administered intratumorally to mice in the HSV1716 and HSV1716 + ibrutinib groups on days 0 and 2. As a control, two 100 µL doses of PBS were administered to mice in the PBS and HSV1716 groups on days 0 and 2. Three days before the first HSV1716 injection, one 25 mg/kg dose of ibrutinib was administered via drinking water to the ibrutinib and HSV1716 + ibrutinib groups. Ibrutinib was administered daily for the duration of the experiment. The PBS and HSV1716 groups received vehicle drinking water containing 1% HP-β-cyclodextrin. Tumors were harvested 72 hours after the first HSV1716 injection. Single cell suspensions of tumor were created by mechanical chopping, followed by incubation in 25 µg/mL liverase blendzyme 3 and 250 µg/mL DNAse I for 1 hour at 37°C. Tumor slurries were passed through a 70 µm cell strainer. 1 x 10^6 cells were blocked with 5% mouse Fc (2.4G2; BD Biosciences) blocking reagent in fluorescent-activated cell sorting (FACS) buffer (1% FBS and 1mM EDTA in PBS). To analyze the immune cells, cells were stained with antibodies against CD206-fluorescein isothiocyanate (FITC) (C068C2), CD11b-Violet 421 (M1/70), CD11c-PerCP/Cy5.5 (N418), F4/80-PE-Cy7 (BM8), Ly-6G-APC-Cy7 (1A8), Ly6c (APC) (AL-21), and CD49b-PE (DX5) on ice for 30 minutes. The staining antibodies were obtained from BioLegend and BD-Biosciences (anti-Ly6C antibody). Stained

cells were washed in FACS buffer and fixed in 0.5% paraformaldehyde. Cellular infiltrate analysis was conducted using a flow cytometry machine and FlowJo software, version 10.03 (Tree Star).

Aim 2: Survival study in A673 Ewing sarcoma model. To determine if the combination of ibrutinib and HSV1716 virotherapy causes tumor regressions and increases survival in Ewing sarcoma, a survival study using A673 xenograft-bearing athymic nude mice was performed. 5.0 x 10^6 A673 cells in a 2:1 150-uL mix of PBS and matrigel were injected subcutaneously into the flanks of athymic nude mice. After tumors reached a volume between 135.4-614.3 mm^3, the mice (n=10) were randomized into one of the four study groups: PBS control, HSV1716, ibrutinib, or HSV1716 + ibrutinib. Most tumors volumes were within a range of 150-250 mm^3. Two 1x10^7 pfu doses of HSV1716 were administered intratumorally to mice in the HSV1716 and HSV1716 + ibrutinib groups on days 0 and 2. As a control, two 100 μL doses of PBS were administered intratumorally to the PBS and HSV1716 groups on days 0 and 2. Three days prior to the first HSV1716 injection, one 25 mg/kg dose of ibrutinib was administered via drinking water to mice in the ibrutinib and HSV1716 + ibrutinib groups. Ibrutinib was administered daily for sixty days. Mice in the PBS and HSV1716 groups were administered vehicle drinking water containing 1% HP-β-cyclodextrin. Mice were weighed once per week, and tumors were measured twice per week. Tumor volume was calculated using the formula: a x b^2 x (π/6), where a is the tumor length and b is the tumor width. Mice were also observed two times per week for signs of endpoint criteria including tumor volume >2,000 mm^3, body weight loss >20%, unusual behavior, and lack of movement. Mice were followed until they reached endpoint criteria or until the conclusion of the study.

Aim 3: Viral replication assay. To determine the effect of ibrutinib on viral replication in HSV1716-treated Ewing sarcoma tumors, serial plaque assays were performed. 5.0 x 10^6 A673 cells in a 2:1 150-uL mix of PBS and matrigel were injected subcutaneously into the flanks of athymic nude mice. After tumors reached a volume between 75.8-798.7 mm^3, the mice (n=5) were randomized into one of two study groups: HSV1716 or HSV1716 + ibrutinib. Most tumor volumes were between 150-350 mm^3. Three days prior to the first HSV1716 injection, one 25 mg/kg dose of ibrutinib was administered daily via drinking water to mice in the HSV1716 + ibrutinib group. Ibrutinib was administered daily for the duration of the experiment. One $1x10^7$ dose pfu of HSV1716 was administered intratumorally to both groups on day 0. Tumors were harvested 3, 72, and 144 hours post HSV1716 injection. Tumors were freeze-thawed three times, and the lysates were collected. 1-4 log serial dilutions in plain DMEM were performed using the lysates. The diluted lysates were used to inoculate 12-well plates of Vero cells for 45-60 minutes before adding 2% carboxymethylcellulose overlay (2% carboxymethylcellulose, 1X MEM, 10% FBS). Vero cells were incubated at 37°C for three days. After three days, the plates were stained with crystal violet for 30 minutes, rinsed with tap water, and dried. To determine viral titer, viral plaques were counted and multiplied by serial dilution.

Aim 4: Survival study in M3-9-M rhabdomyosarcoma model. To determine if the combination of ibrutinib and HSV1716 virotherapy causes tumor regressions and increases survival in rhabdomyosarcoma, a survival study using M3-9-M tumor-bearing B6-albino mice was performed. 5.0 x 10^6 A673 cells in 100µL of PBS were injected subcutaneously into the flanks of B6-albino mice. After tumors reached a volume between 131.2-674.2 mm^3, the mice (n=5-8) were randomized into one of the four study groups: PBS control, HSV1716, ibrutinib, or HSV1716 + ibrutinib. Three $1x10^8$ pfu doses of HSV1716 were administered intratumorally to

mice in the HSV1716 and HSV1716 + ibrutinib groups on days 0, 2, and 4. As a control, three 100 μL doses of PBS were administered intratumorally to mice in the PBS and ibrutinib groups on days 0, 2, and 4. Three days prior to the first HSV1716 injection, one 25 mg/kg dose of ibrutinib was administered via drinking water to mice in the ibrutinib and HSV1716 + ibrutinib groups. Ibrutinib was administered daily for the duration of the experiment. Mice in the PBS and HSV1716 groups were administered vehicle drinking water containing 1% HP-β-cyclodextrin. Mice were weighed once per week, and tumors were measured twice per week. Tumor volume was calculated using the formula: $a \times b^2 \times (\pi/6)$, where a is the tumor length and b is the tumor width. Mice were observed two times per week for signs of endpoint criteria including tumor volume >2,000 mm^3, body weight loss >20%, unusual behavior, and lack of movement. Mice were followed until they reached endpoint criteria.

Results

Ibrutinib reduces MDSC recruitment to the A673 tumor microenvironment. Ibrutinib has been shown in previous studies to selectively reduce MDSC recruitment to the tumor microenvironment via irreversible inhibition of BTK [16]. To determine if ibrutinib reduced MDSC recruitment in the A673 tumor microenvironment, tumor cellular infiltrate analysis was performed using flow cytometry. Ibrutinib reduced the gMDSC and mMDSC populations in tumors treated with ibrutinib compared to those treated with PBS or oHSV alone (Fig. 1). These results confirmed that ibrutinib reduces MDSC recruitment in the A673 tumor microenvironment.

Ibrutinib does not significantly reduce the recruitment of other immune cells to the A673 tumor microenvironment. To determine if ibrutinib impacts the recruitment of other immune cells, we examined its effect on natural killer cells (NK), TAM, M2-like TAM, dendritic cells, and myeloid cells. Tumor cellular infiltrate analysis was performed using flow cytometry. Treatment with ibrutinib did not significantly impact the recruitment of NK, TAM, M2-like TAM, dendritic cells, or myeloid cells to the A673 tumor microenvironment (Fig. 2). The slight, nonsignificant decrease in the myeloid cell population in the ibrutinib treatment group was expected. Because MDSC are one type of myeloid cell, reduction of MDSC will cause a small decrease in the total population of myeloid cells in the tumor cell infiltrate. We also observed a significant difference in TAM frequency between the control group and HSV1716, ibrutinib, and combination groups. This result may be explained by the small sample sizes used. Small sample sizes may also explain the significant difference in M2-like TAM frequency between the control group and the HSV1716 and combination groups. This difference may also be due to treatment

with HSV1716, as there was no difference between tumors treated with PBS or ibrutinib alone. Overall, these results confirm that ibrutinib selectively targets tumor-associated MDSC in A673 tumor-bearing mice and does not significantly affect other immune cell populations.

The combination of ibrutinib and HSV1716 is safe and well-tolerated in tumor-bearing mice. The combination of ibrutinib and virotherapy in mice has not been tested before. To ensure the treatment was safe and did not cause toxicities in tumor-bearing mice, mice were weighed weekly starting the day that ibrutinib/vehicle was first administered. In both the A673 xenograft-bearing athymic nude mice and the M3-9-M tumor-bearing B6-albino mice, no significant changes in weight were seen among any of the treatment groups (Fig. 3). This data serves as a safety measure and demonstrates that the combination of ibrutinib and HSV1716 is safe and well-tolerated in mice bearing A673 and M3-9-M tumors.

Ibrutinib does not improve the efficacy of oncolytic virotherapy in A673 tumor-bearing mice. To assess if ibrutinib enhances the efficacy of oncolytic virotherapy in Ewing sarcoma, we administered 25 mg/kg of oral ibrutinib daily to A673 xenograft-bearing nude mice, along with two intratumoral $1x10^7$ pfu doses of HSV1716 on days 0 and 2. In comparison to the other treatment groups, the combination therapy did not result in a significant increase in animal survival (Fig 4). We also tracked tumor growth by measuring tumors twice per week. Treatment with ibrutinib did not impact established A673 tumor growth in comparison to mice treated with HSV1716 only or PBS. (Fig 5). These results suggests that ibrutinib does not improve the efficacy of HSV1716 virotherapy in A673 Ewing sarcoma xenograft models, and that solely targeting MDSC is not enough to improve the efficacy of oncolytic virotherapy.

Ibrutinib does not enhance HSV1716 viral replication. To investigate the effect of ibrutinib on HSV1716 viral replication, we performed serial plaque assays using A673 tumor lysates. We treated A673 xenograft-bearing mice with daily 25 mg/kg oral doses of ibrutinib, along with one $1x10^7$ pfu intratumoral dose of HSV1716 on day 0. We harvested tumors at 3, 72, and 144 hours post viral infection. Ibrutinib did not have a significant impact on viral replication in A673 xenografts. A673 tumors treated with the combination of ibrutinib and HSV1716 did not show a statistical increase in viral production compared to A673 tumors treated without ibrutinib (Fig 6). The significant difference we found in viral replication between virus-treated tumors at 3 hours, 72 hours, and 144 hours post viral infection was expected. At three hours post infection, virus should be attaching to and infecting tumor cells, so fewer infectious units will be detected. Beyond three hours, we expected to detect more infectious units as the virus lyses tumor cells and replicates in the tumor microenvironment. Overall, the results from this viral replication analysis demonstrate that ibrutinib does not improve HSV1716 replication kinetics in A673 tumors *in vivo*.

Ibrutinib does not improve the efficacy of HSV1716 virotherapy in M3-9-M tumor-bearing mice. After performing the survival study detailed above in immunocompromised mice, we sought to determine if ibrutinib enhances the efficacy of oHSV virotherapy in rhabdomyosarcoma, a more immunogenic model. By using immunocompetent mice, we hoped to be able to assess if the presence of T cells impacted ibrutinib's effect on virotherapuetic efficacy. We administered 25 mg/kg of oral ibrutinib daily to M3-9-M bearing B6-albino mice, along with three $1x10^8$ pfu doses of HSV1716 on days 0, 2, and 4. The combination therapy did not result in a significant increase in animal survival compared to the other treatment groups (Fig

7). We also tracked tumor growth by measuring tumors twice per week. Treatment with ibrutinib did not impact established M3-9-M tumor growth in comparison to mice treated with HSV1716 only or PBS (Fig 8). These results suggests that ibrutinib does not improve the efficacy of HSV1716 oncolytic virotherapy in M3-9-M rhabdomyosarcoma tumor models, and that solely targeting MDSC is not enough to improve the efficacy of oncolytic virotherapy.

Figures

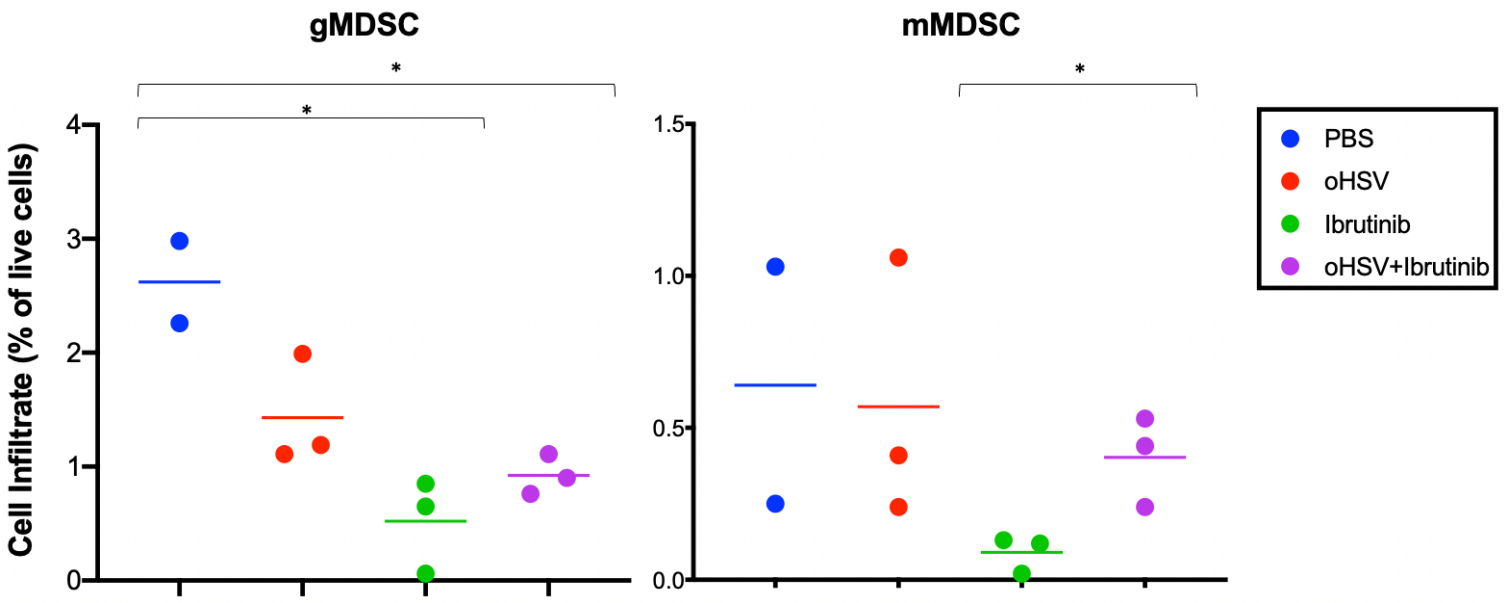

Figure 1. Ibrutinib reduces MDSC recruitment to the A673 tumor microenvironment. Flow cytometry quantification of A673 tumor cellular infiltrate was performed on day 3. * indicates $p<0.05$.

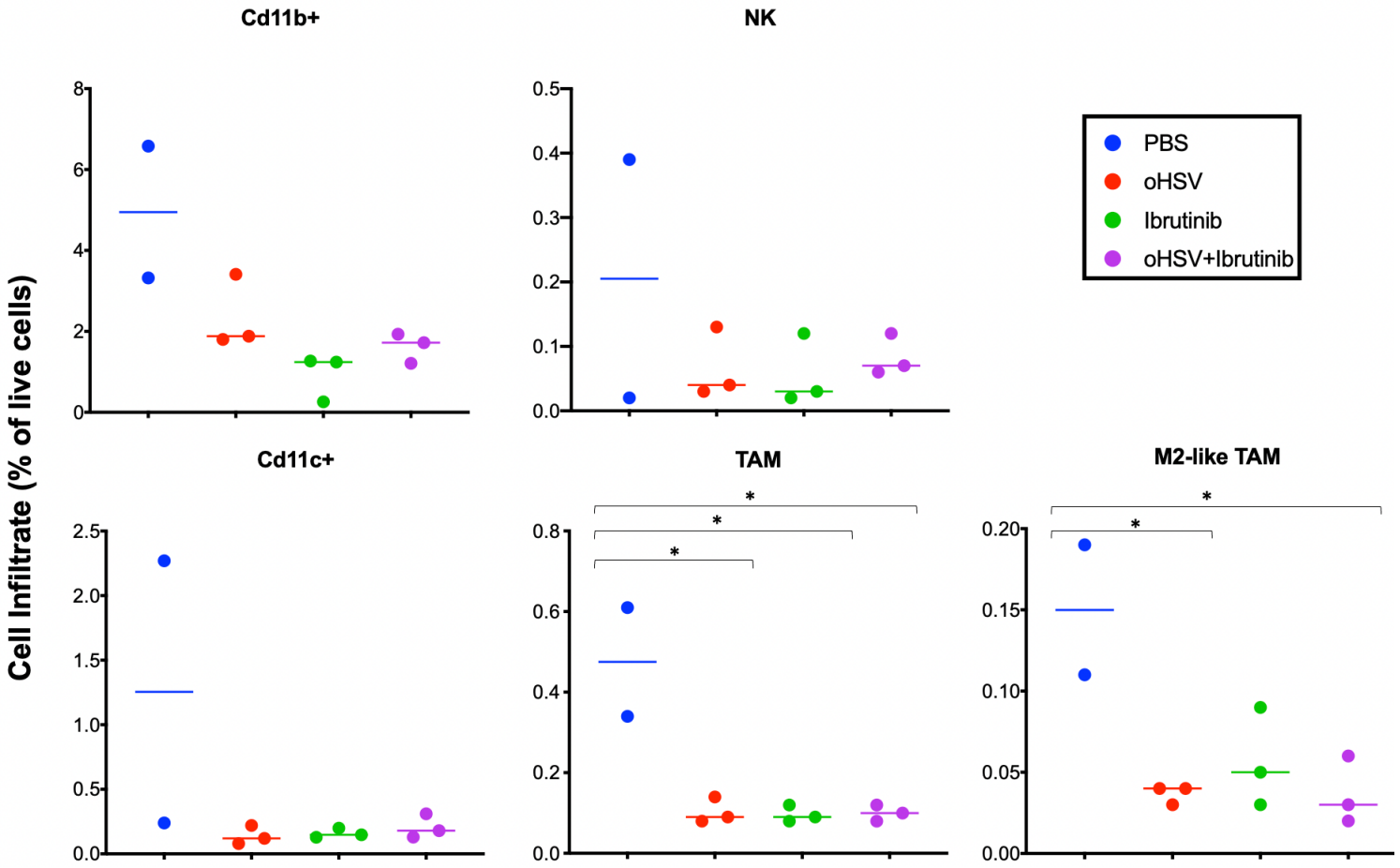

Figure 2. Ibrutinib does not significantly alter the frequency of NK, TAM, M2-like TAM, dendritic cells, or myeloid cells in the A673 tumor microenvironment. Flow cytometry quantification of A673 tumor cellular infiltrates on day 3. * indicates $p<0.05$.

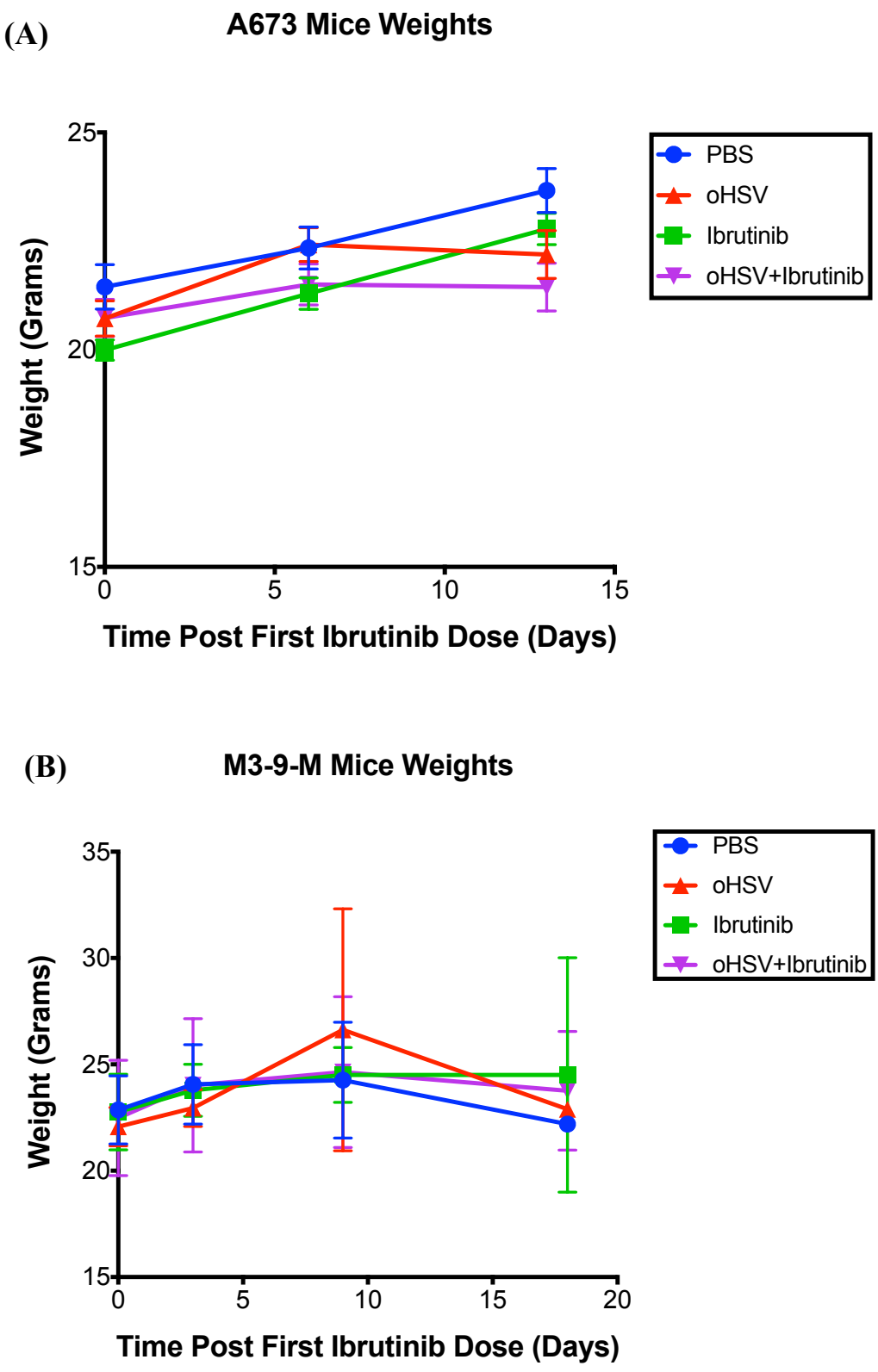

Figure 3. (A) The combination of HSV1716 and ibrutinib is safe and well-tolerated by A673 xenograft-bearing athymic nude mice. **(B)** The combination of HSV1716 and ibrutinib is safe and well-tolerated by M3-9-M tumor-bearing B6-albino mice. Tumor-bearing mice were weighed once a week beginning the first day that ibrutinib was administered. No significant weight change was observed among any of the treatment groups.

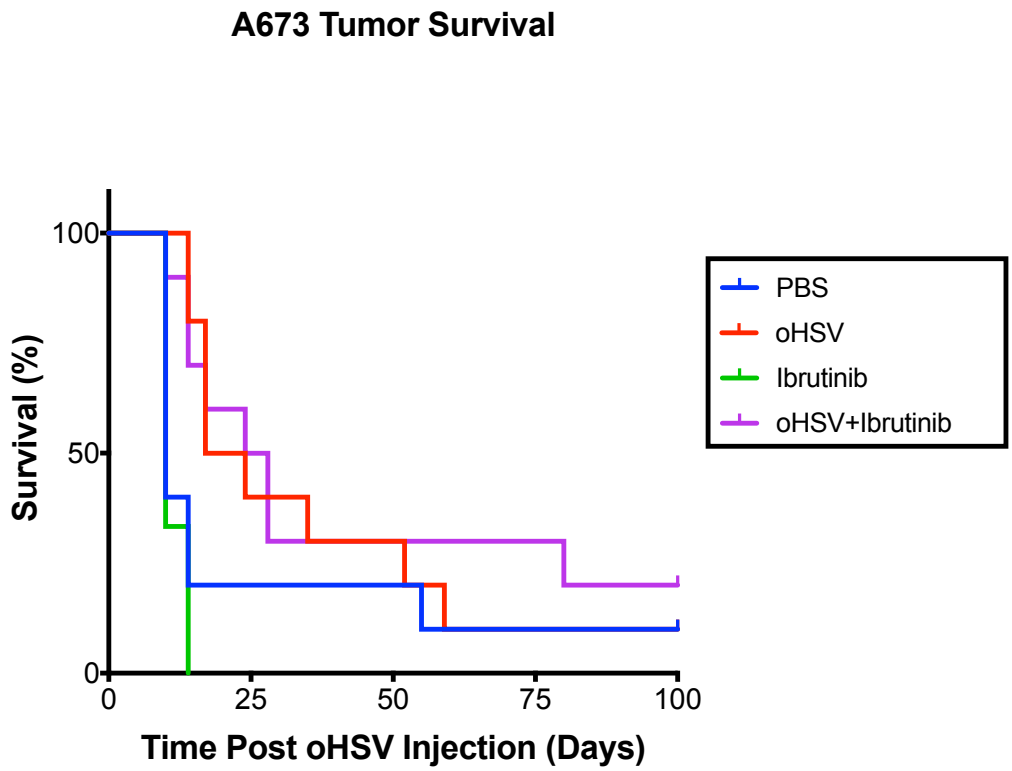

Figure 4. Ibrutinib does not enhance survival in A673 xenograft-bearing athymic nude mice when given alone or combined with HSV1716 virotherapy. A673 flank tumors treated with the regimen described previously were measured until tumor volume reached 2,000 mm^3.

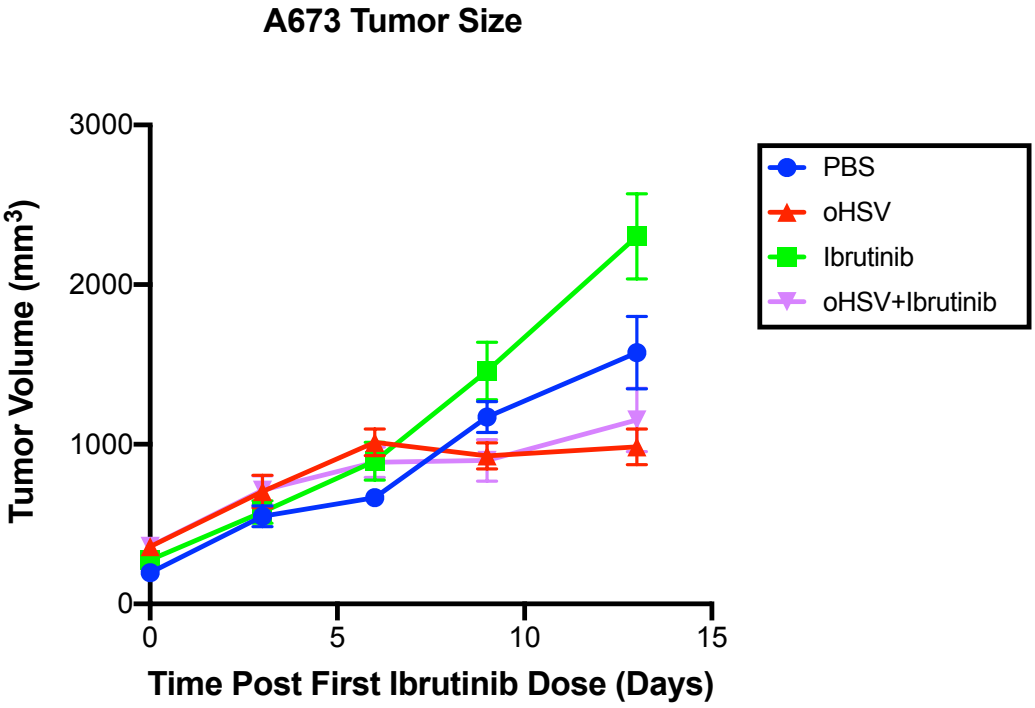

Figure 5. Ibrutinib does not significantly reduce tumor regressions in A673 xenograft-bearing nude mice when given alone or combined with HSV1716 virotherapy. A673 flank tumors treated with the regimen described previously were measured until tumor volume reached 2,000 mm^3.

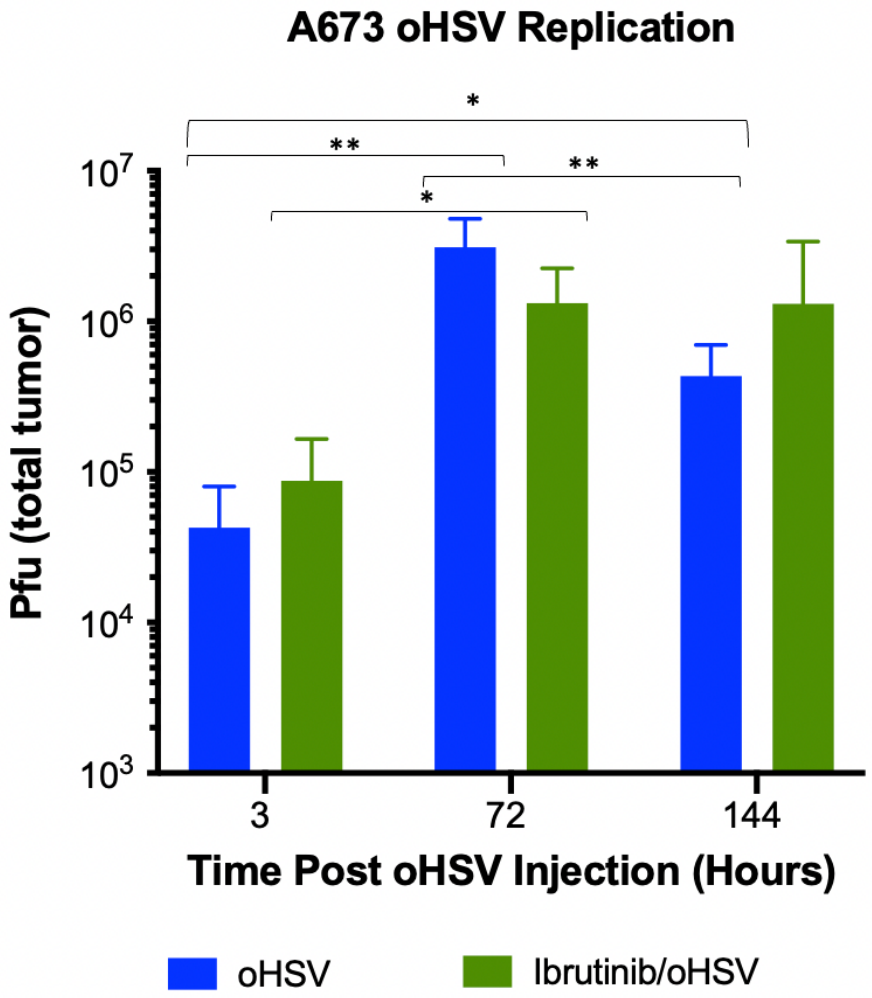

Figure 6. Ibrutinib does not enhance HSV1716 virus replication *in vivo*. HSV1716 virus replication in A673 xenografts treated with one intratumoral $1x10^7$ pfu dose of HSV1716 and with or without ibrutinib. Tumors were harvested 3, 144, and 72 hours post viral infection. Tumors were homogenized, and the lysates were used in serial plaque assays. * indicates $p<0.05$. ** indicates $p<0.005$.

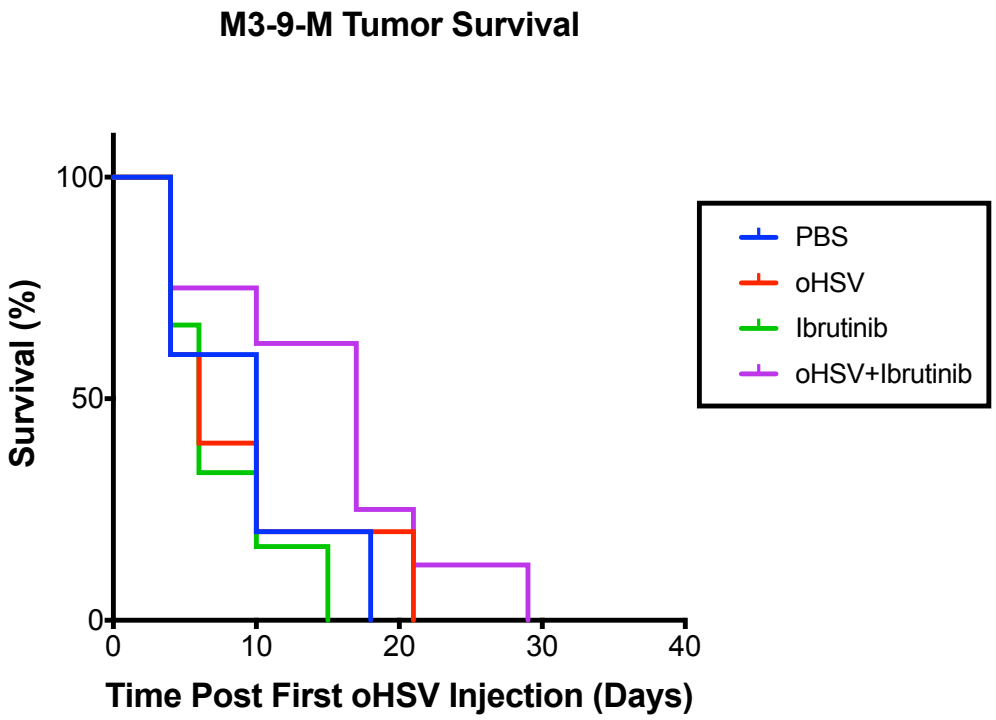

Figure 7. Ibrutinib does not enhance survival in M3-9 tumor-bearing mice when given alone or combined with HSV1716 virotherapy. M3-9-M flank tumors treated with the regimen described previously were measured until tumor volume reached 2,000 mm^3.

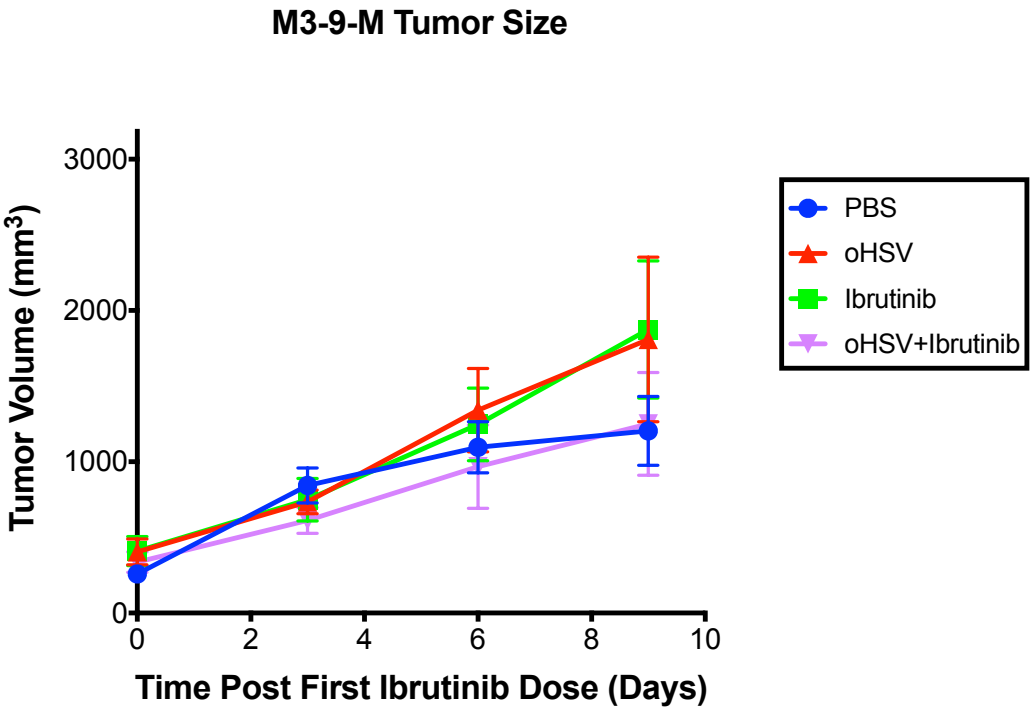

Figure 8. Ibrutinib does not reduce tumor regressions in M3-9-M tumor-bearing mice when given alone or combined with HSV1716 virotherapy. M3-9-M flank tumors treated with the regimen described previously were measured until tumor volume reached 2,000 mm^3.

Discussion

This research investigated the impact of targeting myeloid-derived suppressor cells via BTK inhibition to enhance oHSV virotherapy in pediatric sarcoma models. The immunotherapy drug ibrutinib was chosen for this research because it selectively reduces MDSC recruitment to the tumor microenvironment. The oncolytic virus HSV1716 was selected because clinical trials have shown it to be safe and well-tolerated by pediatric cancer patients.

Using cellular infiltrate analysis, we confirmed our hypothesis that ibrutinib reduces the presence of MDSC in the Ewing sarcoma tumor cellular infiltrate. Ibrutinib significantly reduced tumor-associated gMDSC populations in tumors treated with ibrutinib compared to tumors treated without it. It also reduced tumor-associated mMDSC populations in the ibrutinib treated groups, although this trend did not reach statistical significance. Our results also confirmed that the ibrutinib drinking water was properly formulated and that the drug was active.

Unexpectedly, the results from the cell infiltrate analysis show that oHSV infection did not increase tumor-associated MDSC recruitment, as previous studies have demonstrated. There are multiple possible explanations for these findings. First, a prior study conducted in A673 xenograft-bearing mice utilized the oncolytic herpes virus rRp450 instead of HSV1716 [15]. Different oncolytic viruses have different properties that cause unique therapeutic outcomes [19]. Given this information, it is possible that rRp450 infection significantly increases tumor-associated MDSC recruitment, while HSV1716 has a less significant effect in A673 tumors. Our experiment also used small sample sizes. It should be repeated using larger sample sizes to gain a better understanding of how HSV1716 infection affects A673 tumor-associated MDSC recruitment.

We did not observe any significant impact of ibrutinib on NK, $Cdllc^+$, TAM, M2-like TAM, or $Cd11b^+$ cell populations in A673 tumors. The slight, nonsignificant reduction of $Cd11b^+$ myeloid cells in ibrutinib-treated groups was expected, as MDSC are part of the myeloid cell population. Because ibrutinib decreased the frequency of MDSC, a slight decrease in the total myeloid cell population was also expected. A significant difference was observed in the TAM population between the control group and the HSV1716, ibrutinib, and combination treatment groups. This finding may be explained by the small sample sizes of the experimental groups (n=2-3). In the future, larger sample sizes should be used to better analyze the effect of various treatments on tumor cell infiltrates. Small sample size may also explain the significant difference in M2-like TAM frequency between the control group and the HSV1716 and combination treatment groups. This difference could also be due to HSV1716 treatment, as there was no difference between the control and ibrutinib treatment groups.

We next sought to test our hypothesis that ibrutinib enhances HSV1716 replication *in vivo*. We found that ibrutinib does not impact viral replication in HSV1716-treated A673 tumors. This suggests that targeting tumor-associated MDSC does not influence HSV1716 replication kinetics. We did observe a significant difference in viral replication between HSV1716-treated tumors at 3, 72, and 144 hours post infection. These results were expected because at three hours post infection the virus should be attaching to and infecting tumor cells. In tumors harvested 3 hours post infection, we calculated an average viral titer between $1x10^4$-$1x10^5$ pfu. We administered a $1x10^7$ pfu dose of virus, which means that at 3 hours post infection, 99 percent of the virus was infecting tumor cells. By 72 hours, the virus should have lysed open tumor cells and began replication, so more infectious units will be observed. This effect was sustained at 144 hours post injection because we used tumor-bearing athymic nude mice. While these mice have

NK cells and macrophages that attack virus-infected cells, they lack T cells. Without T cells, the immune response to virus-infected cells is less effective, which leads to sustained viral replication at 144 hours post infection. In immunocompetent mice, we typically observe a loss of viral replication by one week post infection [17].

Because the combination of HSV1716 and ibrutinib has not been tested in animal models before, we sought to confirm that the treatment regime was safe *in vivo*. To test this, we weighed mice once per week beginning the day that ibrutinib was first administered. No significant change in weight was observed in any of the treatment groups in both athymic nude mice and B6-albino mice, which confirms that the combination of ibrutinib and HSV1716 is safe and well-tolerated by tumor-bearing mice.

To evaluate the effect of ibrutinib on oHSV virotherapy, we performed a survival study in A673 xenograft-bearing athymic nude mice. Treatment with ibrutinib alone or in combination with HSV1716 did not result in increased survival or impact tumor growth. Although we hypothesized that ibrutinib would enhance the efficacy of oHSV therapy by reducing tumor-associated MDSC recruitment, our findings do not support this idea. The results suggest that ibrutinib does not improve the efficacy of oHSV virotherapy, and that targeting MDSC alone is not enough to enhance oHSV virotherapy.

The lack of therapeutic efficacy we observed in the athymic nude mice could be due to the absence of T cells. Ibrutinib has been reported to reduce the suppression of T cell proliferation in the tumor area by targeting MDSC. Because athymic nude mice lack T cells, some of the efficacy of ibrutinib may be lost in these animal models. Additionally, numerous studies have shown that the antitumor responses initiated by oHSV therapy may be dependent on T cell activity. In one study, the combination of HSV1716 and A8301, a transforming growth

factor beta receptor 1 (TGF-βR1) inhibitor, significantly prolonged survival in M3-9-M tumor-bearing mice. This efficacy was completely lost, however, when the therapy was tested in athymic nude mice [18]. Given this information, our findings support the idea that antitumor T cell activity is necessary to improve virotherapeutic efficacy.

We next tested our hypothesis that ibrutinib enhances oncolytic virotherapy in a more immunogenic mouse model. M3-9-M, a murine rhabdomyosarcoma cell line, was chosen because it responds well to HSV1716 infection and can be used in immunocompetent mice [17]. Ibrutinib also failed to enhance oncolytic virotherapy in M3-9-M tumor-bearing B6-albino mice. It did not improve survival or impact tumor growth when administered alone or in combination with HSV1716. This data suggests that solely targeting tumor-resident MDSC is not enough to improve the therapeutic efficacy of oHSV virotherapy.

One factor that may have influenced the results of the M3-9-M survival study was the rapid growth of the M3-9-M tumors. We began administering ibrutinib three days prior to viral infection. By the time we administered the first dose of virus, many of the tumors had volumes greater than 150-250 mm^3. Because most tumors were larger than 150-250 mm^3 when virus treatment began, it may have been too late to observe any therapeutic effect from the combination of HSV1716 and ibrutinib. When using this tumor model in future studies, we will monitor tumor progression more closely and consider administering ibrutinib more than three days prior to viral infection, so we can observe its full therapeutic effect.

In the future, we plan to test a triple combination therapy of ibrutinib, oHSV, and anti-PD-1 antibody in M3-9-M tumor-bearing mice. A prior study found that ibrutinib improved the efficacy of anti-PD-L1 therapy in Balb/c mice bearing EMT6 mammary carcinomas. The combination of ibrutinib and anti-PD-L1 therapy resulted in significant tumor reduction

compared to either treatment alone. The combination treatment also produced more complete responses compared to anti-PD-L1 therapy alone [16]. Additionally, a previous Cripe lab study found that the combination of anti-PD-1 antibody and HSV1716 significantly prolongs survival in M3-9-M tumor-bearing mice [17]. Taken together, these findings suggest that the triple combination of ibrutinib, anti-PD-1 antibody, and HSV1716 will enhance survival via several different mechanisms. These mechanisms include suppressing MDSC recruitment, reducing MDSC suppression of T cell proliferation, preventing T cell exhaustion, and inducing T cell recruitment in the tumor microenvironment. We plan to perform a pilot survival study to test whether the combination of ibrutinib, oHSV, and anti-PD-1 antibody enhances survival in M3-9-M tumor-bearing mice compared to the combination of oHSV and anti-PD-1 treatment. We will continue to use weight as a safety measure to ensure that other combination therapies are safe for tumor-bearing mice. We will also monitor the growth of M3-9-M tumors closely and consider administering ibrutinib earlier than three days before oHSV infection.

The present research found that while ibrutinib does reduce tumor-associated MDSC recruitment, it does not enhance oHSV virotherapy in A673 and M3-9-M pediatric sarcoma models *in vivo*. Additionally, it does not improve HSV1716 replication in A673 tumors *in vivo*. The current findings suggest that solely targeting tumor-resident MDSC is not sufficient to improve the efficacy of oHSV virotherapy. More research must be conducted to fully understand the relationship between the tumor microenvironment and oncolytic virotherapy. The influence of the tumor microenvironment on the efficacy of oncolytic virotherapy will continued to be investigated in future studies with the ultimate goal of creating targeted myelolytic-virotherapy treatments that limit long term- side effects and improve outcomes for pediatric sarcoma patients.

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